http://dx.doi.org/10.22161/ijeab/3.5.23 *ISSN: 2456-1878*

Effect of some biocontrol agents against root-knot nematode (*Meloidogyne incognita* race2)

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Abstract— *Culture filtrate of four rhizospheric fungi and four biocontrol agents were studied in vitro for their efficacy against Meloidogyne incognita race 2. The per cent mortality and egg hatching inhibition was proportional to the concentration of culture filtrate and the duration of exposure period. Culture filtrates of Trichoderma viride, Trichoderma harzianum, Trichoderma sp., Fusarium sp., Penicillium sp. and Aspergillus sp. significantly induced inhibition of egg hatching and mortality of Meloidogyne incognita race 2.The highest percentage of inhibition of egg hatching and juvenile mortality was recorded in Trichoderma harzianum followed by Trichoderma viride and Trichoderma sp.*

Keywords— Biocontrol agent, culture filtrate, egg hatching, juvenile mortality, root-knot nematode.

I. INTRODUCTION

Root-knot nematode (*Meloidogyne* spp.) is an important plant pathogen affecting crop production throughout the world. Since, indiscriminate use of nematicides is responsible for environmental and human health concerns; the search for new microbial strains as nematode control agents is relevant. As fungi cohabit together with nematodes in the rhizosphere, their toxic metabolites may be responsible for keeping a low level of nematode populations [1]. The search for nematotoxic or antagonistic compounds in culture filtrates has greatly intensified in recent years, due to the number of toxins, enzymes or compounds derivable from their metabolites [2-7]. Assays with culture filtrates may provide first information about the role of a fungus in the plant rhizosphere, as *in vitro* studies showed toxic and inhibitory effects of several filtrates toward plant parasitic nematodes [8]. Toxic effects of fungal culture filtrates on *M. incognita* have been studied by several workers [9-16] and had showed different levels of efficacy [17-20]. Due to the differences of soil ecological types and climate, a broad range of fungi remains far unexplored. Therefore, present study was made to isolate rhizospheric fungal associations of root knot nematode infected plants and evaluate the potential of some isolated fungi and already recognized biocontrol agents (against insect pests and diseases) on hatching of eggs and mortality of second-stage juveniles of *Meloidogyne incognita* race 2 *in vitro*.

II. MATERIALS AND METHODS

Collection of samples

Soil samples were collected in different localities around Jorhat, Assam comprising an area approximately 1000 ha, in order to identify the root-knot nematode infection. To isolate the fungal antagonists from rhizosphere soils of infested cucurbits, tomato, brinjal, okra, cabbage, citrus, banana and tea, a total of 100 soil (500 g each) were collected. Samples were stored at 15^0C for not more than one week.

Fungal isolation and identification

Soil mycoflora was isolated by serial dilution pour plate technique [21, 22]. One g of rhizosphere soil was dispensed in 9 ml sterile water, from the 10^{-5} dilution, 50 µl were inoculated over Petri plates containing PDA media. The plates were incubated at room temperature 24±2°C for 48 hrs. Materials of the pure culture were mounted in Lactophenol, stained with Cotton blue and the morphological observations of hyphae, sporangiophore/conidiophores and conidia were done with the help of a Compound light microscope at 400X magnification [23, 24]. Axenic cultures of the fungi were obtained by single spore isolations [25] and the cultures were maintained on PDA slants. *Trichoderma viride*, *Trichoderma harzianum*, *Beauveria bassiana*, *Metarhizium anisopliae* were procured from the Department of Plant Pathology, AAU, Jorhat, Assam.

Nematode inoculum and mass culturing

The inoculum of root-knot nematode *M. incognita* race 2 was collected from naturally infested tomato crop in field and single egg mass was used to raise pure culture. Mass culturing of nematodes was done on tomato variety Sel 7, in order to get regular supply of the inoculums for the experiment. One month old tomato seedlings were

http://dx.doi.org/10.22161/ijeab/3.5.23 *ISSN: 2456-1878*

inoculated with small volume of egg suspension approximately consisting of 2000 eggs of *M.incognita* race 2. These pots were watered and kept in glasshouse at temperature 28-35°C.

Preparation of fungal culture filtrates

To evaluate the nematicidal potential of the cell free fungal culture filtrate the most frequently occurring isolates belonging to the genera of *Trichoderma, Aspergillus, Penicillium and Fusarium* were selected. *Beauveria bassiana, Metarhizium anisopliae, Trichoderma viride and Trichoderma harzianum* were procured from the Department of Plant Pathology, AAU, Jorhat, Assam. These strains were inoculated on to Petri plates containing Potato Dextrose Agar medium and incubated for 7 to 10 days at 27°C. From these actively growing cultures, one disc each of 0.5 cm diameter was transferred to 250 mL Erlenmeyer flask containing 50 mL Potato Dextrose broth. These flasks were incubated at 27±1°C for 15 days. The culture was filtered through two layers of Whatman filter Paper No.1. Filtrates thus obtained were designated as standard solution (100%). Different dilutions (50%, 25%, and 10%) of each fungal filtrate were prepared by adding required amount of sterilized distilled water.

Hatching test

To determine the effect of culture filtrate on the hatching of eggs of *M. incognita* sterilized Petri dishes of 5 cm dia were separately pipette two ml of culture filtrate. Five sterilized healthy egg masses of nearly uniform size of *M.incognita* were transferred to each dish. The egg masses placed in culture medium served as control. All Petri dishes were kept at 28±2°C in completely randomized design, replicated thrice. Observations were recorded on every 24 h interval up to 72 h with the aid of stereomicroscope. The per cent egg hatch was calculated by the following formula and mean of three replications was presented in Table.2.

$$\text{Hatching \%} = \frac{\text{No. of hatched juveniles}}{\text{No. of hatched + unhatched eggs}} \times 100$$

Mortality of second stage juveniles (J2)

For determining the effect of fungal filtrates on juvenile mortality of *M.incognita* race 2, egg masses were collected from an infested root and allowed to hatch in distilled water with aeration. The hatched J_2 were collected in a beaker. One hundred freshly hatched second stage juveniles were transferred to 5 cm dia Petri dishes containing 2 ml filtrates of different dilutions of each fungus and medium separately. Equal number of J_2 was also transferred to separate Petri dishes containing culture medium to serve as control. Petri dishes were kept at 28±2°C temperature in completely randomized design, replicated thrice. Observation on the number of dead J_2 for every 24, 48 and 72 h of exposure was recorded with the aid of stereomicroscope and per cent mortality of juveniles was calculated. The J_2 were considered dead when they did not move when probing with a fine needle Mean percentage of dead J_2 was estimated using the following formula and presented in the Table 3.

$$\text{Per cent mortality} = \frac{\text{Total number of dead juveniles}}{\text{Total number of juveniles}} \times 100$$

STATISTICAL ANALYSIS

Per cent egg hatch and per cent mortality data was subjected to statistical analysis using the three factorial completely randomized design statistical package. The critical differences in main effects i.e. isolates, concentration, and time of exposure as well as in their interactions were tested at P=0.05.

III. RESULTS

A total of four isolates of different genera of fungi were isolated from the soil rhizosphere of *M.incognita* race 2 infected plants. *Trichoderma sp. Fusarium* sp., *Aspergillus* sp. and *Penicillium* sp. was isolated from rhizospheric soil of banana, cowpea, brinjal and cucumber respectively.

The results presented in Table 1 revealed significant differences among isolates (biocontrol agent) (T), concentration of culture filtrate(C) and exposure period(t). The culture filtrate of *Trichoderma harzianum* followed by *T.viride, T.sp. Aspergillus* sp., *P.* sp. and *F.*sp. adversely affected the larval hatching of *M.incognita* race 2. Irrespective of concentration of culture filtrate (C) and time of exposure period (t), *T.harzianum* was the most effective bioagent followed by *T.viride* and *T.*sp. as the hatching of *M.incognita* was suppressed. Similarly, irrespective of isolate (biocontrol agent) (T) and concentration of culture filtrate (C), time of exposure(t) also affected the larval hatching. With increase in exposure period up to 72 hours there was a correspondingly increased in egg hatching. With increase in the dilution of culture filtrate, the cumulative hatching was increased irrespective of isolate (T) and time of exposure period (t). Highest inhibition in hatching was obtained in 100% concentration of each fungal culture filtrates. The percentage hatching of *M.incognita* was 18.35% during 72 h exposure in the 100% concentrations of culture filtrates of *Trichoderma viride* followed by *T.harzianum* with percentage hatching 20.36%. *Beauveria bassiana* and *Metarhizium anisopliae* showed negligible

http://dx.doi.org/10.22161/ijeab/3.5.23 *ISSN: 2456-1878*

effect on inhibition of egg hatching of *M.incognita*. Hatching percentage was 59.34%, 58.35% respectively at 10% concentration of culture filtrate during 72 h exposure period.

The data showed in the Table 2 revealed that all the culture filtrates of isolates were having nematicidal effect of varying degree on *M.incognita* race 2. Per cent mortality of nematodes was directly proportional to the concentration of culture filtrate and the period of exposure. Irrespective of concentration of culture filtrate (C) and duration of exposure(t), six isolates namely *Trichoderma harzianum, Trichoderma viride, Trichoderma* sp., *Fusarium* sp., *Aspergillus* sp. and *Penicillum* sp. were exhibited nematicidal effects on *M. incognita* J_2. The activity of *Trichoderma harzianum* was the highest, with juvenile mortality 82.66%, 85.33%, 89.33% for 24, 48 and 72 h, respectively at 100% concentration of culture filtrate. This was followed by *T.viride* with juvenile mortality 79.00%, 81.66%, 85.66% for 24, 48 and 72 h, respectively at 100% concentration of culture filtrate. All the new isolate namely *Trichoderma* sp., *Penicillum* sp., *Aspergillus* sp., *and Fusarium* sp. displayed more than 50% juvenile mortality during 24 h exposure time at 25% concentration of culture filtrate. On the other hand, *Beauveria bassiana* and *Metarhizium anisopliae* showed the lowest toxicity, caused only 67.66% and 51.65% juvenile mortality at 50% concentration of culture filtrate during 72 h exposure time.

IV. DISCUSSION

Culture filtrates of many fungi possess nematicidal activity against nematodes, due to the production of toxic metabolites [26].Variable effect of fungal filtrates on hatching and mortality of root-knot nematode *M.incognita* race 2 observed in the present study may be attributed to the varied nature of toxic metabolites produced by different fungi. Species of *Trichoderma, Fusarium, Paecilomyces, Aspergillus, Penicillium* are known to produce toxins and antibiotics like viridian, fusaric acid, lilacin, oxalic acid and penicillic acid [27, 28]. Various mechanisms have been suggested for the biocontrol activity of *Trichoderma* spp. against phytopathogenic fungi: antibiosis, competition, mycoparasitism, and enzymatic hydrolysis [29, 30]. *Trichoderma* spp. is utilized in the production of a number of antibiotics, such as trichoderin, trichodermol A and harzianolide. *Trichoderma* produces molecules such as 6-pentyl α-pyrone, VOCs and enzymes [31] that can attack the cuticle of nematodes. Also, its hyphae form a physical barrier, which is a difficult step for nematodes, since the fungus grows along with the plant roots. Successful parasitism of the nematode by *Trichoderma* requires mechanisms to facilitate penetration of the nematode cuticles or eggshells. The involvement of lytic enzymes has long been suggested and demonstrated in *Meloidogyne* parasitism [32]. Besides direct antagonism, other mechanisms involved in *Meloidogyne* control by *Trichoderma* spp. include production of fungal metabolites and induced resistance [33-35]. *Trichoderma harzianum* has been found to be an effective biocontrol agent for the management of root-knot and other nematodes [36-39]. Direct interactions between *T. harzianum* and the potato cyst nematode *Globodera rostochiensis* were demonstrated *in vitro* by Saifullah and Thomas [40].The fungus penetrated the cysts and the eggs in those cysts, resulting in larval death. *Beauveria bassiana* has a repressive action on nematodes of the genus *Meloidogyne* spp. [41-47]. *B. bassiana* may have more than a single bioactive metabolite that are responsible for nematicidal activities, and each metabolite may act on a different site. Ghayedi and Abdollahi [48] purified the isolated fungus and also they showed the biocontrol potential of the isolate on *Heterodera avenae*, with 47.1% of larval mortality. Chen *et al*., [49] found that *B. bassiana* showed little parasitism of nematode eggs but reduced hatch of *Heterodera glycines*. Studies have shown that *Beauveria* can produce beauvericin and oosporin. Beauvericin has a weak activity against *M. incognita* [50-53]. The percentage mortality and inhibition of hatching of root-knot nematode were directly proportional to the concentration of culture filtrates of *B.bassiana* [54]. Biocontrol potential of *M. anisopliae* against some species of root knot nematodes has been shown [55-58]. The lethal effect of *M. anisopliae* culture extract has been also reported [59].Some species of *Metarhizium* has root colonization ability [60, 61]. Some isolates of *M. anisopliae* have endophytic behavior [62]. The fungus produces sticky conidia that attach to nematode cuticle [63]. The conidia germinate, parasitize and kill the cadaver, by direct penetration and producing the infective hyphae inside the nematode body. The fungus produces some cyclopeptides and destruxins which may play an important role in its pathogenicity [64]. Prior to any direct attack to the host, the fungus produces destruxin A and destruxin B that can kill the host [65].

V. CONCLUSION

It is clear from this work that, in plant rhizosphere, there are many fungi that have potentialities for controlling root-knot nematode. Among four fungal isolates and four bioagents, *Trichoderma harzianum* was exhibited the highest

http://dx.doi.org/10.22161/ijeab/3.5.23 *ISSN: 2456-1878*

production of nematicidal activities against root-knot nematode (*M. incognita* race 2) *in vitro*.

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http://dx.doi.org/10.22161/ijeab/3.5.23 *ISSN: 2456-1878*

Table.1: Effect of culture filtrate of some fungal bioagent on egg hatching of Meloidogyne incognita race 2.

Isolate(T)	Culture filtrate concentration (C) (%)	Period of exposure(t)			Isolate (T) Mean	Culture filtrate concentration (C) Mean
		24h	48h	72 h		
Trichoderma sp.	10	24.35(29.53)	32.33(34.64)	40.35(39.38)	24.19 (29.06)	40.11(39.22)
	25	20.34(26.79)	28.34(32.14)	34.35(35.84)		34.82(35.93)
	50	16.33(23.70)	22.35(28.17)	26.32(30.86)		28.32(31.74)
	100	10.30(18.66)	12.30(20.53)	22.65(28.41)		20.91(26.39)
Penicillium sp.	10	28.32(32.11)	34.30(35.82)	46.36(42.89)	25.34 (30.33)	
	25	20.35(26.79)	30.65(33.62)	38.32(38.21)		
	50	18.34(25.15)	24.30(29.54)	28.30(32.15)		
	100	10.30(18.71)	14.32(22.20)	20.30(26.73)		
Aspergillus sp.	10	28.30(32.06)	34.33(35.85)	43.34(41.16)	24.74 (29.92)	
	25	22.35(28.17)	28.34(32.04)	38.35(38.22)		
	50	16.35(23.70)	24.35(29.49)	26.33(30.86)		
	100	10.33(18.66)	12.30(20.49)	22.32(28.19)		
Fusarium sp.	10	26.32(30.81)	34.30(35.85)	44.36(41.74)	26.16 (30.53)	
	25	22.32(28.17)	30.35(33.37)	38.32(38.23)		
	50	16.30(23.73)	24.35(29.54)	28.35(32.14)		
	100	12.32(19.91)	14.32(22.20)	22.34(28.17)		
Trichoderma viride	10	25.33(30.18)	30.30(33.41)	42.30(40.58)	23.74 (28.63)	
	25	20.34(26.78)	28.35(32.08)	36.32(37.05)		
	50	12.30(20.53)	22.32(28.19)	26.35(30.85)		
	100	8.30(16.53)	14.35(22.20)	18.35(22.44)		
Trichoderma harzianum	10	22.30(28.17)	30.30(33.40)	36.34(37.05)	22.00 (27.59)	
	25	18.34(25.12)	26.30(30.85)	30.35(33.36)		
	50	14.35(22.19)	20.35(26.77)	22.35(28.19)		
	100	10.35(18.66)	12.35(20.42)	20.36(26.79)		
Beauveria bassiana	10	33.60(35.45)	42.32(40.58)	59.34(50.39)	33.53 (35.12)	
	25	28.30(32.11)	38.32(38.23)	46.32(42.89)		
	50	24.32(29.54)	34.30(35.85)	32.30(34.64)		
	100	18.62(25.33)	20.30(26.77)	24.34(29.53)		
Metarhizium anisopliae	10	33.65(35.45)	42.32(40.58)	58.35(49.79)	33.60 (35.16)	
	25	30.32(33.40)	36.32(37.05)	46.34(42.89)		
	50	26.30(30.85)	32.35(34.64)	32.35(34.64)		
	100	16.30(23.70)	20.35(26.77)	28.36(32.12)		
Culture broth	10	51.00(45.57)	74.34(59.57)	84.37(66.70)	64.41 (53.74)	
	25	50.00(44.98)	72.65(58.52)	79.38(62.96)		
	50	48.33(44.04)	67.34(55.15)	77.30(61.58)		
	100	45.00(42.12)	55.65(48.24)	67.65(55.34)		
Period of Exposure (t) Mean		23.36(28.27)	31.19(33.47)	38.60(38.22)		
CV=6.92 CD(P=0.05): Treatment(T):1.06; Concentration(C):0.71; Period of exposure(t):0.61; T ×C: 2.13;T×t: 1.84; C×t:1.23;T×C×t: 3.69						

Figures in the parentheses are Arc-Sine transformed values

http://dx.doi.org/10.22161/ijeab/3.5.23 *ISSN: 2456-1878*

Table.2: Effect of culture filtrate of some fungal bioagent on juvenile mortality of Meloidogyne incognita race 2.

Isolate (T)	Culture filtrate concentration (C) (%)	Period of exposure(t)			Isolate (T) Mean	Culture filtrate concentration (C) Mean
		24h	48h	72 h		
Trichoderma sp.	10	44.66(41.92)	47.62(43.65)	51.60(45.95)	65.11 (54.08)	45.00(41.98)
	25	61.60(51.75)	65.64(54.14)	71.66(57.86)		56.98(49.07)
	50	63.62(52.94)	69.65(56.62)	77.64(61.80)		60.72(51.39)
	100	68.65(55.97)	77.66(61.86)	81.33(64.42)		66.09(54.96)
Penicillium sp.	10	38.67(38.42)	44.66(41.93)	50.60(45.38)	55.36 (48.13)	
	25	51.66(45.95)	54.66(47.67)	57.66(49.40)		
	50	54.66(47.67)	59.62(50.57)	61.60(51.75)		
	100	57.33(49.22)	65.60(54.13)	67.64(55.39)		
Aspergillus sp.	10	41.67(40.19)	50.66(45.37)	53.66(47.09)	62.71 (52.52)	
	25	60.34(50.96)	63.66(52.93)	67.66(55.34)		
	50	61.34(51.56)	65.66(54.14)	69.66(56.62)		
	100	67.33(55.24)	73.66(59.14)	77.33(61.56)		
Fusarium sp.	10	39.33(38.82)	43.66(41.35)	49.66(44.80)	63.71 (53.23)	
	25	60.33(50.98)	67.66(55.36)	69.66(56.65)		
	50	64.66(53.54)	69.33(56.38)	75.66(60.47)		
	100	69.66(56.58)	75.33(60.36)	79.66(63.44)		
Trichoderma viride	10	54.00(47.29)	59.66(50.65)	61.66(51.74)	71.77 (58.22)	
	25	67.33(55.15)	69.66(56.69)	73.66(59.13)		
	50	74.00(59.35)	77.66(61.81)	77.33(61.59)		
	100	79.00(62.75)	81.66(64.69)	85.66(67.75)		
Trichoderma harzianum	10	55.33(48.05)	57.66(49.42)	61.66(51.78)	72.25 (58.69)	
	25	69.35(56.38)	69.60(56.68)	71.60(57.86)		
	50	77.60(61.81)	71.33(57.80)	75.65(60.47)		
	100	82.66(65.42)	85.33(67.67)	89.33(70.95)		
Beauveria bassiana	10	32.66(34.83)	41.60(40.19)	55.66(48.25)	51.67 (46.03)	
	25	45.30(42.31)	46.66(43.08)	59.64(50.61)		
	50	47.33(43.46)	51.30(45.76)	67.66(55.35)		
	100	49.65(44.79)	55.00(47.86)	67.62(55.79)		
Metarhizium anisopliae	10	32.00(34.43)	37.00(37.41)	43.66(41.35)	45.50 (42.40)	
	25	42.33(40.58)	45.66(42.50)	49.65(44.78)		
	50	43.00(40.96)	47.62(43.64)	51.65(45.95)		
	100	46.30(42.89)	49.64(44.80)	57.60(49.40)		
Culture broth	10	14.00(21.83)	24.00(29.23)	28.30(31.93)	26.72 (30.86)	
	25	18.00(24.93)	28.00(31.93)	30.00(33.14)		
	50	22.00(27.95)	30.00(33.19)	32.40(34.39)		
	100	24.00(29.32)	34.00(35.65)	36.00(36.82)		
Period of Exposure (t) Mean		52.26(46.29)	57.18(49.34)	62.19(52.42)		
CV=5.83, CD(P=0.05): Treatment(T):1.33; Concentration(C):0.88; Period of exposure (t): 0.76; T×C: 2.66; T×t: 2.30; C×t:1.53; T×C×t: 4.60						

Figures in the parentheses are Arc-Sine transformed values